

POLLEN SIRING SUCCESS IN THE CALIFORNIA WILDFLOWER  
*CLARKIA UNGUICULATA* (ONAGRACEAE)

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ABSTRACT

Cryptic self-incompatibility (CSI) is a type of non-random mating observed in self-compatible plants in which outcross pollen sires proportionally more seeds than self pollen when both pollen types are available on the stigma. Levels of CSI are known to vary among individuals and populations. We conducted competitive pollinations consisting of mixtures of self and outcross pollen to investigate reports of CSI in certain populations of *Clarkia unguiculata* Lindl. We also investigated how the order of self and outcross pollen deposition on the stigma influences the degree of nonrandom mating. Finally, we looked at whether the source population of outcross pollen affected the ability of outcross pollen to outcompete self pollen. We utilized recessive (white-petaled) maternal (and self pollen donor) plants from a locality near Morro Bay, California, and dominant (pink-petaled) outcross pollen donor plants from 17 localities widespread through the species range in California. Progenies from pollinations made with equal mixtures of self and cross pollen included significantly more outcross-pollinated than self-pollinated offspring in 10 of the 17 cross-pollen donor populations. However six populations showed no significant difference between self- and outcross-pollinated offspring, and one population yielded a majority of progeny sired by self pollen. Progenies from sequential self pollen followed by outcross pollinations included significantly more self offspring in 12 of the donor populations, no significant difference between outcross and self offspring in four donor populations and significantly more outcross offspring in one donor population. Progenies from sequential outcross pollen followed by self pollinations included significantly more outcross offspring in 15 donor populations and no significant difference between outcross and self offspring in two donor populations. Our results confirm the occurrence of non-random mating in *C. unguiculata*, and demonstrate that the degree of non-random mating can depend on the order of self vs. outcross pollen deposition and the source population of outcross pollen. This non-random mating can influence the proportion of self and outcross progeny in sequential pollinations.

Key Words: *Clarkia unguiculata*, cryptic self-incompatibility, geitonogamy, non-random mating, sexual conflict.

Cryptic self-incompatibility is essentially a case of non-random mating in which normal seed set occurs upon self pollination in the absence of competitive outcross pollen. However, with mixtures of self and outcross pollen, weak self rejection reactions promote preferential fertilization by outcross pollen (Bateman 1956; Weller and Ornduff 1977; Eckert and Allen 1997; Kruszewski and Galloway 2006).

Nonrandom mating in plants is not limited to examples of CSI but also occurs in mixtures of outcross pollen from several donors, as in *Raphanus sativus* L. (Marshall and Ellstrand 1986; Marshall 1991, 1998). Of particular interest in these cases is the observation that pollen donors may differ significantly in their ability to sire seed, and the rank order of their siring ability can be consistent across several maternal plants

(Marshall 1998). This fact suggests that the source of outcross pollen is likely to affect the degree of non-random mating due to CSI.

Self-incompatibility may evolve in outcrossing populations in response to inbreeding depression. If a normally outcrossing population carries a genetic load, self-incompatibility could confer a fitness advantage to maternal plants if they produce more outcross progeny than selfed progeny in mixed pollinations. In contrast to “complete” self-incompatibility, plants exhibiting CSI maintain the ability to produce progeny through self-fertilization. This ability should confer the fitness advantage of reproductive assurance to annual plants that grow where access to pollinators or mates is limited. During growing seasons when pollinators and other resources are plentiful, non-random mating by favoring outcross pollen allows maternal plants to increase fitness by increasing the proportion of outcross progeny they produce. During less optimal growing seasons, when resources and

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pollinators are limiting, maternal fitness is enhanced through self-pollination.

Another important consideration is the role that non-random mating by favoring outcross pollen might play in promoting outcross pollination in plants with varied patterns of pollen deposition. The number of pollen grains deposited on the stigma in natural populations of *Clarkia unguiculata* Lindl. increases with time (Nemeth and Smith-Huerta 2003), indicating that pollinators make multiple visits to flowers, depositing loads of pollen sequentially over time. These loads of pollen are likely to vary in composition from mixtures of outcross pollen to loads of self pollen. Geitonogamous self pollination is possible since *C. unguiculata* is strongly protandrous (Vasek 1968, 1977; Vasek et al. 1987). A pollinator could visit a flower in the male phase and then visit another flower on the same plant in the female phase resulting in self pollination. It is possible that CSI could promote outcross pollination in cases where the stigma of a flower first receives a load of self pollen and soon after receives a load of outcross pollen. In fact, although geitonogamy is possible (and probable) in *C. unguiculata*, outcrossing rates in a natural population have been measured at greater than 90% (Vasek 1965). Non-random mating by favoring outcross pollen (CSI) may well have contributed to these high outcrossing rates. In fact, CSI is known to occur in at least one population of *C. unguiculata* (Bowman 1987) and in one population of *C. gracilis* A. Nelson and J. F. Macbr. (Jones 1994). Interestingly, CSI was reported to be completely absent from another population of *C. unguiculata* (Travers and Mazer 2000). This suggests that the strength and nature and of non-random mating could vary from population to population and also with the source of outcross pollen.

The purpose of our investigation was to examine a single population of *C. unguiculata* maternal plants to determine whether the order that self and outcross pollen is deposited on the stigma affects non-random mating. Furthermore, we examined if the source population of outcross pollen affects levels of CSI. This closer examination of non-random mating in *C. unguiculata* is important given the possibility of sequential and geitonogamous pollination in *C. unguiculata* (Vasek 1968, 1977; Vasek et al. 1987; Nemeth and Smith-Huerta 2003), the variation in levels of CSI documented previously in two different populations of *C. unguiculata* (Bowman 1987; Travers and Mazer 2000), and that pollen donors may differ significantly in their ability to sire seed (Marshall 1998).

## MATERIALS AND METHODS

Mixed-donor pollinations were employed to determine the relative frequency of fertilization

by self vs. cross pollen. Test plants were genetically marked for petal color. Flower color in *C. unguiculata* varies from white to red or purple but most populations are characterized by pink or lavender-pink flowers. White flowers are conditioned by a single recessive allele and pink or red flowers are conditioned by a dominant allele at the same locus (Rasmuson 1920; Vasek 1965, plus extensive unpublished data; Bowman 1984). Plants with pink flowers contain malvidin 3, 5-diglucoside in their flowers, leaves, stems and seedlings (Bowman 1987) whereas plants with white flowers lack this and other anthocyanins. Consequently, flower color can be determined in the seedling stage merely by scoring seedlings as green (non-anthocyanous) or red (anthocyanous) because green seedlings grow up to produce white flowers and red seedlings grow up to produce pink flowers (Bowman 1987; Vasek 1965).

## Organization of Experiments

Ten white-flowered families were developed from available stocks from a natural population near Morro Bay, California because they were available and we knew they were fully self-fertile (Vasek 1986). Each family consisted of approximately 6–8 siblings (homozygous for white petal) and was used as a line of maternal test plants, and simultaneously as self-pollen donors.

Seventeen pink-flowered families were developed from available stocks from Morro Bay and 16 widespread localities in California (Table 1). Each family consisted of about 6–8 siblings and was used as a line of cross pollen donors. These plants were homozygous for pink flowers because they were grown from selected, available stocks known to have produced only pink-flowered progeny.

Seeds from the source localities were sown on vermiculite in December, 1987 in a University of California greenhouse in Riverside, California. Subsequently, seedlings were transplanted to 6 inch pots of standard UC soil mix, irrigated as needed, fertilized weekly with half strength Hoagland's solution and grown to maturity in the same greenhouse. Experimental pollinations were made during a period of about two months in the spring of 1988.

## Mixed-Donor Pollinations

All experimental pollinations utilized pollen from a pink-flowered cross-pollen donor and pollen from a white-flowered self pollen donor. The self pollen donor was also the seed parent. Pollen was always applied to stigmas which were 1–2 days old as judged by the degree of stigma expansion not greater than 180 degrees (see figure 2 in Smith-Huerta and Vasek 1984).

TABLE 1. SEED COLLECTION LOCALITIES (ALL IN CALIFORNIA) FOR CROSS-POLLEN DONOR PLANTS. DIR = direction from Morro Bay: S = Coast Ranges to the south; N = Coast Ranges to the north; E = Sierra Nevada to the East; DIS = approximate linear distance in km from Morro Bay.

Population	Locality	County	DIR	DIS
15	Morro Bay - Atascadero Rd	San Luis Obispo	—	00
4	Santa Maria	Santa Barbara	S	58
5	Pinnacles Natl. Monument	San Benito	N	126
11	Rancheria Rd	Kern	E	185
6	Kern River	Kern	E	188
13	Caliente Hills, Low Canyon,	Kern	E	189
17	Caliente Hills, High Canyon	Kern	E	190
8	Santa Paula	Ventura	S	193
7	Sequoia	Tulare	E	220
9	Bouquet Canyon	Los Angeles	S	238
14	Laurel Canyon	Los Angeles	S	260
2	Jackson	Amador	E	330
3	Riverside -Fairmount Park	Riverside	S	350
10	San Luis Rey	San Diego	S	398
16	Old Castle Rd	San Diego	S	406
1	Clear Lake	Lake	N	430
12	Feather River, North Fork	Butte	E	485

One protocol (MIX) followed the methodology used by Bowman (1987). Cross pollen and self pollen were mixed together in equal amounts and then applied to the stigma of the maternal test plant, which was also the self-pollen donor. In the second protocol (S/X), self pollen was applied first followed immediately by cross pollen. In the third protocol (X/S), cross pollen was applied first, followed immediately by self pollen.

We pollinated 246 flowers in 82 competitive pollinations. Each pollination included one pollination by each of the three protocols described above. Several siblings of each cross pollen donor population were used, for a total of 47 pollen donors from the 17 cross-pollen donor lines. Thus, the actual competitive comparisons involved closely related siblings rather than identical individuals.

Following competitive pollinations, the resulting capsules were harvested at maturity, the seeds were then separated, counted, weighed and stored over the summer.

#### Data Harvest

Beginning in October 1988, seeds were sown on vermiculite in small plastic pots and put in a temperature controlled chamber. Temperatures were approximately 20 degrees C with a 12 h day 12 h night lighting schedule. Two weeks after germination, seedlings were moved to a greenhouse for continued development because space in the temperature controlled chamber was limited. Red pigments in stems and along leaf veins develop well with cool temperatures and bright daylight. Our greenhouse conditions were not always optimum for red pigment development (e.g., warm weather). Consequently, if any seedlings were not clearly red or not clearly green

they were grown to maturity for direct scoring of flower color.

#### Analysis

We expect the progeny from competitive (MIX) pollinations to include half pink-flowered outcrosses if mating is non-random. However, preferential functioning of either cross or self pollen will yield ratios of white to pink progeny significantly different from 1:1. The frequency of outcrosses in each resulting progeny was multiplied by 100 and expressed as a percent. The progeny of (S/X) pollinations should include significantly more self pollinated white than pink progeny if mating is random. We expect the first pollen grains on a receptive stigma should interfere with the normal germination of pollen arriving later. The progeny of (X/S) pollinations should include significantly more pink than white progeny.

Each of the 246 progenies from MIX, S/X and X/S pollinations was tested for significant departure from an expected 1:1 ratio of outcrosses to selfs by a G-test (Zar 1984). A Bonferroni P-value correction was applied to avoid inflated type-I error (avoid high risk of false positive results) across the multiple tests (Zar 1984). Progenies from all of the competitive pollinations within the 17 populations were also tested for significant departure from an expected 1:1 ratio of outcross to selfs by a G-test (Zar 1984).

#### RESULTS

Both the order of pollen deposition and source of outcross pollen had variable effects on the degree of non-random mating due to CSI in our competitive pollinations (Table 2, Fig. 1). In the

TABLE 2. G-TEST VALUES FOR ALL PROGENY PRODUCED IN COMPETITIVE POLLINATIONS. The symbols “\*,” “\*\*,” “\*\*\*,” “\*\*\*\*,” and “ns” indicate significant difference from a 1:1 ratio of self to outcross progeny at the 5%, 1%, and 0.1% levels, and not significantly different, respectively.

Population	MIX	S/X	X/S
1	68.219***	48.753***	61.926***
2	16.934***	0.134 ns	9.150**
3	34.464***	1.590 ns	51.451***
4	17.357***	0.0055 ns	71.653***
5	32.142***	19.534***	55.477***
6	12.659***	29.500***	46.019***
7	9.419**	11.027***	16.933***
8	14.710***	37.016***	42.659***
9	7.905**	80.327***	2.987 ns
10	7.266**	12.162***	14.009***
11	1.697 ns	29.354***	30.372***
12	2.337 ns	7.703**	24.856***
13	0.032 ns	26.640***	23.774***
14	0.643 ns	9.550**	25.641***
15	0.0303 ns	144.658***	3.412 ns
16	0.024 ns	0.468 ns	13.832***
17	11.512***	106.878***	9.662***

MIX competitive pollinations, 10 of the 17 cross pollen donor populations exhibited CSI, yielding progenies that significantly favored outcross pollen (Table 2, Fig. 1). However six of the 17 pollen donor populations did not exhibit CSI, and one population yielded a majority of progeny sired by self pollen (Table 2, Fig. 1).

In the S/X competitive pollinations, one of the 17 cross pollen donor populations yielded progenies that significantly favored outcross pollen, 12 pollen donor populations significantly favored self pollen, and four of the donor populations did not differ significantly from a 1:1 ratio (Table 2, Fig. 1).

Fifteen of the pollen donor populations in X/S competitive pollinations significantly favored outcross pollen, and two donor populations did not differ significantly from a 1:1 ratio (Table 2, Fig. 1).

## DISCUSSION

Overall, the results of our MIX pollinations confirm the occurrence of CSI and non-random mating in *Clarkia unguiculata*. More than half of the competitive MIX pollinations yielded progenies that significantly favored outcross pollen, and approximately 20% of competitive pollinations yielded progenies with significantly more offspring produced by self pollen. Only about 23% of competitive pollinations yielded progenies that did not differ from a 1:1 ratio. Our investigation differs in detail and scope from previous studies of CSI and non-random mating in *Clarkia* (Bowman 1987; Jones 1994; Travers and Mazer 2000), and may help to explain their variable results. Previous studies were conducted

within single populations of plants (Jones 1994; Travers and Mazer 2000) or used a commercial seed source (Bowman 1987). Our study included maternal plants derived from one population and outcross pollen plants derived from 17 populations distributed throughout California. In the present study, competitive pollinations made with pollen from 10 of the 17 populations yielded results similar to those found by Bowman (1987) and Jones (1994) with a majority of pollinations favoring outcross pollen. Competitive pollinations with outcross pollen derived from six of the populations yielded results similar to those found by Travers and Mazer (2000), with outcross pollen favored in fewer than half the pollinations. In contrast to all of the previous studies, competitive pollinations made with outcross pollen derived from 1 of our study populations yielded a majority of progenies in which self pollen was favored significantly over outcross pollen.

Non-random mating when outcross pollen is favored over self pollen has the potential to reduce the negative effects of inbreeding depression in populations through the increased production of outcross progeny, at the same time preserving the ability of individuals to produce offspring by selfing. Inbreeding depression has been documented in several populations of *C. tembloriensis* Vasek (Holtsford and Ellstrand 1990) and the magnitude of this inbreeding depression was shown to vary between populations. It is possible that populations of *C. unguiculata* could vary with respect to genetic load, and selective pressure promoting non-random mating favoring self pollen might vary between populations. This could account for the variation observed in non-random mating in previous studies (Bowman 1987; Jones 1994; Travers and Mazer 2000).

As stated above, non-random mating by favoring outcross pollen not only promotes the production of outcross progeny, but also preserves the ability of a plant to produce offspring through self pollination. This provides reproductive assurance to annual plants that grow where access to mates may be limiting. Numbers of individuals in populations of *C. unguiculata* can vary from only a few plants to thousands of individuals (Lewis and Lewis 1955; Vasek 1964) and those growing in more marginal areas of the species range may experience large seasonal fluctuations in population size (Lewis and Lewis 1955). During seasons when plant populations are small, pollinators are rare, and access to mates limited, fitness might be enhanced by the production of self progeny.

Our sequential pollinations address the question of whether non-random mating by favoring outcross pollen can somehow mitigate the effects of geitonogamy when self pollen arrives first on

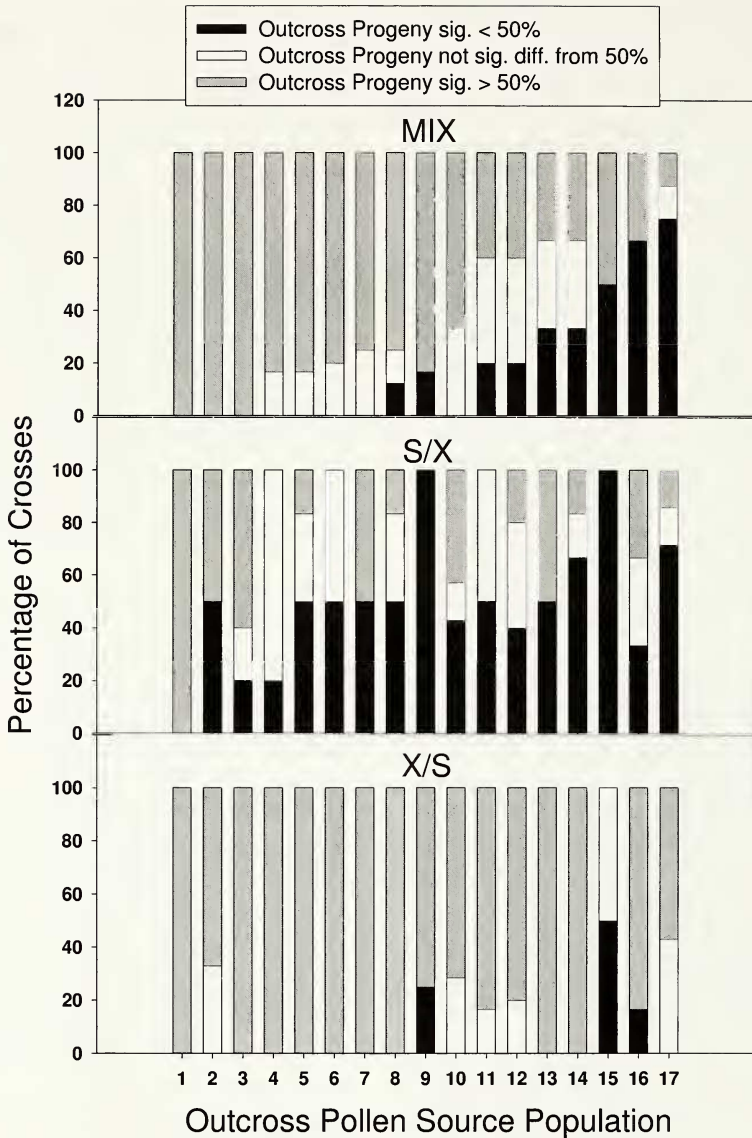


FIG. 1. Percent of total competitive pollinations within each of 17 cross pollen donor populations that yielded outcross progenies either significantly more or significantly less than expected by chance, or yielded outcross progeny not significantly different from chance expectations. For the MIX treatment, cross and self pollen were mixed together in equal amounts and then applied to the stigma of the maternal test plant, which was also the self-pollen donor. For S/X, self pollen was applied first followed immediately by cross pollen. For X/S, cross pollen was applied first, followed immediately by self pollen.

the stigma followed by outcross pollen. Rates of geitonogamy are influenced by the number of flowers that are open simultaneously on a plant (Karron et al. 2004). *Clarkia unguiculata* is strongly protandrous (anthers shed pollen before the stigma becomes receptive), and may have as many as 12 open flowers per spike (Vasek 1968, 1977; Vasek et al. 1987). Anthers of individual flowers shed pollen for up to 11 days before the stigma becomes receptive (Vasek 1968, 1977; Vasek et al. 1987). If pollinators visit more than one flower per plant, geitonogamous self polli-

nation is likely. Geitonogamy does not provide reproductive assurance and is considered to be "an unavoidable by-product of selection for outcrossing success" in plants that have numerous flowers that are open simultaneously in an inflorescence (Goodwillie et al. 2005). It is possible that non-random mating by favoring outcross pollen could mitigate the unavoidable selfing that occurs when pollinators visit more than one flower per plant in *C. unguiculata*, and our S/X sequential pollinations were designed to explore this idea. If mating is random, S/X

pollinations (outcross pollen grains deposited immediately after self pollen grains) should yield progenies with significantly more self than outcross offspring. Twelve of the donor populations did yield progenies that significantly favored self pollen, even though six of these populations displayed non-random mating in MIX pollinations. In these cases, the documented presence of non-random mating did not appear to mitigate the effects of geitonogamy. In contrast, S/X sequential pollinations in five of the donor populations did not favor self progeny. Of these, four of the populations produced progeny that did not differ from a 1:1 ratio of self to outcross, and one population significantly favored outcross pollen. With one exception (population 16), these were the donor populations that displayed the strongest non-random mating by favoring outcross pollen in MIX pollinations. It appears that strong non-random mating by favoring outcross pollen has the potential to mitigate the effects of geitonogamy when self pollen arrives first on the stigma followed by outcross pollen.

Our X/S pollinations explored further the interactions of non-random mating and geitonogamy in *Clarkia*. The X/S pollinations were expected to produce significantly more outcross than self progeny in all pollinations, since the advantage of outcross pollen arriving first on the stigma should be enhanced by non-random mating. This proved to be the case in all but two of the donor populations which showed no significant difference between the number of self and outcross progeny produced. Interestingly, these two donor populations were the only populations in which self pollen was significantly favored in all of their sequential S/X pollinations.

The physical mechanism responsible for differential siring success in non-random mating and CSI has been investigated in other studies (Hessing 1989; Weller and Ornduff 1989; Aizen et al. 1990; Eckert and Allen 1997; Kruszewski and Galloway 2006; Figueroa-Castro and Holtsford 2009). In all of the above investigations, with one exception, outcross pollen germinated faster on the stigma and grew faster through the style than self pollen. The single exception occurred in *Campanulastrum americanum* Small in which pollen tube growth rates did not differ between self and outcross pollen (Kruszewski and Galloway 2006). Although we did not measure pollen germination and tube growth rates in the present study, this was the focus of previous studies in both *C. unguiculata* (Nemeth and Smith-Huerta 2002; Smith-Huerta et al. 2007) and *C. tembloricensis* (Smith-Huerta 1996; Kerwin and Smith-Huerta 2000). Similar to *Campanulastrum americanum* (Kruszewski and Galloway 2006), no difference in percent germination or rate of pollen tube growth was observed between self and

outcross pollen in single donor pollinations in these two *Clarkia* species (Smith-Huerta 1996; Kerwin and Smith-Huerta 2000; Nemeth and Smith-Huerta 2002). It is possible that self and outcross pollen must be present together on the stigma and in the style for the non-random mating of CSI to occur. This appears to be the case in *Clarkia*. Pollen germination was significantly reduced in two donor pollinations of self + outcross pollen and in outcross + outcross pollen (from two different donors) in *C. unguiculata* (Nemeth and Smith-Huerta 2002). Further, germination of pollen decreased with increasing contact between pollen grains (Nemeth and Smith-Huerta 2002). Pollen-pollen interactions, mediated by the stigma, might provide a possible mechanism to explain the differences in the relative success of self and outcross pollen observed in reported non-random mating and CSI in *Clarkia*.

The present investigation goes beyond an examination of self vs. outcross pollen performance within a single population, and examines the performance of outcross pollen derived from foreign populations. These outcross pollen source populations occur from 58 to 485 km from the maternal (self pollen) population. Clearly, the maternal plants in our study would not normally encounter outcross pollen from these populations. Interestingly, the Morro Bay population, which provided all of our maternal plants, did not show CSI in MIX crosses when Morro Bay plants were the source of outcross pollen. In contrast, levels of CSI could be very high when pollen from foreign populations was used. This great difference in outcross pollen donor success suggests that pollen-pistil interaction may evolve differently in each population of *C. unguiculata*, as a result of sexual conflict between male and female function. In plants, sexual conflict occurs when optimal reproductive fitness strategies for pollen differ from those of the maternal plant. The trait of CSI has the potential to enhance the fitness of ovules but not of pollen, thus creating a male-female sexual conflict. Although sexual conflict has been studied mostly in animals, several recent studies have examined the occurrence of sexual conflict in plants (Prasad and Bedhomme 2006; Lankinen and Larsson 2009; Madjidian and Lankinen 2009). In one instance, similar to the present study, male-female interactions were investigated in cross pollinations between plants derived from 4 different populations of *Collinsia heterophylla* Graham (Madjidian and Lankinen 2009). In this plant, the onset of stigma receptivity may be affected by both the source and recipient of the pollen, with early receptivity and fertilization resulting in the production of fewer seeds than late receptivity. In experimental cross pollinations, it was found that pollen donors from foreign populations were

more successful at inducing stigma receptivity than pollen donors derived from the same population (Madjidian and Lankinen 2009). These results may be interpreted to suggest that there is “sexually antagonistic coevolution” between maternal plants and pollen within populations of *C. heterophylla* (Madjidian and Lankinen 2009). It appears that when maternal plants receive pollen from foreign populations they are somehow “released from the cost of local pollen” (Madjidian and Lankinen 2009). In the present study, CSI and non-random mating varied extensively depending on the population source of outcross pollen. It is possible that this reflects a similar “release from the cost of local pollen” in our experiments.

In sum, we document the occurrence of non-random mating and CSI in crosses between different populations of *Clarkia unguiculata*, and demonstrate that this non-random mating can also influence the proportion of self and outcross progeny in sequential pollinations. The non-random mating observed in CSI may promote outcrossing in protandrous plants subject to geitonogamous pollination and contribute to reproductive assurance when access to mates is limited. Finally, the fact that levels of CSI vary between sources of pollen donors suggests that sexual conflict between pollen and maternal plants may result in coevolution unique to each population of *C. unguiculata*.

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